Wheat Polyphenol Oxidase: Distribution and Genetic Mapping in Three Inbred Line Populations

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ABSTRACT

The enzyme polyphenol oxidase (PPO) has been implicated in discoloration of Asian noodles. The recombinant inbred line (RIL) populations, M6/'Opata 85', NY18/CC, and ND2603/'Butte 86' were used to investigate the distribution, chromosome location, and number of loci involved in wheat (*Triticum aestivum* L.) PPO. PPO activity was measured by means of the substrates L-DOPA (L-3,4-dihydroxyphenyl-alanine) and L-tyrosine. The M6/Opata 85 RIL population had a normal distribution, while the ND2603/Butte 86 RIL population had a bimodal distribution for PPO activity (L-DOPA assay). Transgressive segregants were observed for all three populations. Correlations between L-DOPA and L-tyrosine assays for PPO activity were low to medium and could be attributed to substrate specificity and environment. For the combined analysis of M6/Opata 85 RIL populations, the QTL marker *Xfba314* (located on chromosome 2D) showed significant association with PPO activity for the L-DOPA assay. For the combined analysis of NY18/CC, three QTL markers for L-DOPA, and two different QTL markers for L-tyrosine, revealed an association with PPO activity at LOD scores of >2.4. The QTL markers for the NY18/CC RIL population were located on chromosomes 2A, 2B, 3D, and 6B. The ND2603/Butte 86 population had relatively few other loci for linkage analysis and only the marker *Xbcd907.RV.I* located on chromosome 3BS showed a weak association with PPO activity on the basis of the L-DOPA assay. The identified QTL markers will be useful for marker-assisted selection as they build upon the evolving maps for these populations, and for resolving in greater detail the genetic basis of PPO activity in wheat.

NOODLES ARE STAPLE FOODS in many Asian countries. The two popular classes of Asian noodles are white-salted and yellow alkaline (Nagao, 1996). A clear bright yellow color is preferred for alkaline noodles. However, in many samples, there is a time-dependent darkening of Asian noodles that is undesirable. Cantonese noodles which are sold fresh are especially sensitive to the darkening problem. Polyphenol oxidase (PPO, EC 1.10.3.1) plays a major role in time-dependent darkening of noodles and other wheat products (Baik et al., 1995; Hatcher et al., 1999; Kruger et al., 1994; Miskelly, 1996; Morris et al., 2000). Most of the wheat seed PPO activity is located in bran, especially in the aleurone layer (Sullivan, 1946). Phenolic acids such as ferulic, sinapic, and vanillic, which are potential substrates for PPO, are endogenous to the wheat plant and grain (Hatcher and Kruger, 1997). PPO is believed to be involved in oxidation of such phenolic acids to quinones, and the quinones in turn react with amines and thiol groups or undergo selfpolymerization to produce dark or brown products. Milling wheat grain at a higher flour extraction rate increases the darkening effect (Baik et al., 1994; Hatcher and Kruger, 1993). Flour protein content had a negative association with flour PPO activity (Park et al., 1997) presumably because of reactivity of phenolic side groups.

Polyphenol oxidase activity varies among wheat cultivars and is also affected by environment (Baik et al., 1994; Park et al., 1997). Durum wheat (*Triticum durum* Desf.) cultivars generally have very low PPO activity, whereas hexaploid wheat cultivars vary in the amount of PPO activity (Baik et al., 1995; Kruger et al., 1994; Lamkin et al., 1981; Miskelly, 1996). A significant difference was also observed in the distribution of PPO among flour millstreams of Canadian wheat market classes (Hatcher and Kruger, 1993). According to Park et al. (1997) variation in wheat flour PPO activities among growing locations was greater than variation among genotypes. Large seed-to-seed variation was observed for PPO activity in spring wheat and triticale (× *Triticosecale* Wittmack) cultivars with L-tyrosine and catechol as substrates (McCaig et al., 1999).

PPO genes have been cloned and sequenced in several plant species, including sugar cane (*Saccharum* spp. hybrids) (Bucheli et al., 1996). Seven PPO genes have been identified for tomato (*Lycopersicon esculentum* Mill.) (Thipyapong et al., 1997) and 12 isozymes of PPO have been reported for wheat (Kruger, 1974). There is no wheat PPO DNA sequence available so far, but on the basis of the number of isozymes reported, it can be safely assumed that most, if not all, hexaploid wheat genotypes will likely have more than one PPO gene.

It has been demonstrated that molecular markers may be used to explain a proportion of the phenotypic variance for complex characters (Tanksley, 1993). In wheat, a QTL marker has been identified for PPO activity in a recombinant inbred line population derived from a cross between NY18 and CC (Udall, 1997). The restriction fragment length polymorphism (RFLP) marker *Xcdo373*, located on wheat chromosome 2 (chromosome arm not mentioned), accounted for over 40% of the variation of PPO activity in all regression models

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Abbreviations: AU, absorbance units; CC, Clark's Cream; RFLP, restriction fragment length polymorphism; L-DOPA, L-3,4-dihydroxyphenyl alanine; NY18, NY6432-18; LOD, $\log_{10}$ (probability of linkage/probability of random assortment); PPO, polyphenol oxidase; QTL, quantitative trait loci; RILs, recombinant inbred lines.

indicating the possible presence of a gene affecting PPO. Genetic linkage of wheat seed PPO activity with chromosome 2D has been suggested on the basis of analysis of nullisomic-tetrasomic lines (Anderson and Morris, 2001). A study by Jimenez and Dubcovsky (1999) also showed that genes located in homoeologous group 2 of the wheat chromosome play a major role in PPO activity. Although the homoeologous group 2 chromosomes have been implicated, there is no definite agreement on the exact chromosome location of the wheat PPO gene(s). The objectives of this study were to use L-DOPA (diphenol) and L-tyrosine (monophenol) assays and molecular markers to: (i) investigate the distribution of PPO activity in three wheat RIL populations, and (ii) determine the chromosome location, and number of loci for PPO activity in three wheat RILs.

MATERIALS AND METHODS

Plant Materials and Growing Conditions

In all cases, fields were monitored for disease and insect problems, and none of these affected quality of the grain. Populations were grown in suitable locations for acceptable grain quality.

M6/Opata 85

One hundred twelve F_2-derived recombinant inbred lines (RILs) in F_{12} generation were developed by single seed descent from a cross between the *T. aestivum* synthetic ('M6', 'Altar 84'/*Aegilops squarrosa* accession 219, experimental designation CIGM86.940) and Opata M85. This is the population used by the International Triticeae Mapping Initiative (ITMI) to construct a dense linkage map in wheat (Van Deynze et al., 1995). Three lines (17, 18, and 33) were deleted from the statistical and mapping analyses as they were indicated to be different from the original (Mark Sorrells and Calvin Qualset, 2001, personal communication). The RILs and the two parents were grown in two replications near Tule Lake, CA, during the 1997 and 1998 growing seasons. Plots were planted in March and harvested in August in both years. Crop management practices were standard for wheat at this location and included 300 kg ha^{-1} ammonium sulfate and supplemental irrigation. The soil type was Tulebasin mucky silty clay loam with 130 g kg^{-1} organic matter. The average monthly precipitation from May to August was 2.08 cm in 1997 (with a range of 0.43 cm in July to 2.90 cm in June) and 3.15 cm (with a range of 0.0 cm in August to 10.9 cm in May) in 1998.

NY18/CC

This population consisted of 78 F_5-derived RILs developed by single-seed descent from the cross NY18/CC (Anderson et al., 1993). NY18 is a soft white wheat with good soft wheat quality characteristics and CC is a hard white wheat that has a high level of seed dormancy. The RIL populations plus parents were grown in three environments. The 1994 growouts were conducted at the Ohio State University, Ohio Agricultural Research and Development Center (OSU-OARDC) at Wooster, OH. Seeds in the greenhouse were planted in October 1993, vernalized for 8 wk at 4°C, and transplanted in December of 1993. Greenhouse growing conditions were 21 ± 1°C day, 15 ± 1°C night with a 16-h photoperiod. Ten plants of each RIL were transplanted into 15-cm clay pots (one plant per pot) containing an equal soil mix of Wooster silt loam soil, sand, peat and perlite. Plants were fertilized once weekly with a standard greenhouse nutrient solution and harvested during March-April, 1994. Each RIL was bulk-harvested across the 10 pots. The population was also grown in the field at Wooster, OH on the OSU-OARDC Snyder Farm. Plots were planted in single rows, 0.3 by 1 m in October, 1994 and hand-harvested in July, 1995. The soil type was Wooster silt loam (Typic Fragiudalf, fine, loamy). Crop management practices were standard for wheat at this location. The average monthly precipitation from May to August 1995 was 9.4 cm (with a range of 5.16 cm in May to 14.05 cm in August). The third growout was at Central Ferry, WA, in the 1997-1998 growing season. Plots were planted in October, 1997 and harvested in July, 1998. The soil type for Central Ferry was fine silty, mixed, mesic Typic Natrixeroll. Crop management practices were standard for wheat at this location. The average monthly precipitation from May to August was 1.35 cm (with a range of 0.43 cm in August to 1.93 cm in May and June). Supplemental irrigation was provided.

ND2603/Butte 86

One hundred thirty-four F_5-derived RILs were developed by single-seed descent from a cross between ND2603/Butte 86. Butte 86 is a hard red spring wheat cultivar adapted to the northern Great Plains. ND2603 is a hard red spring wheat breeding line selected from the cross 'Sumai 3'/'Wheaton'. Sumai 3 is a donor of type 2 resistance to Fusarium head blight (caused by *Fusarium graminearum* Schwabe). Two replications of the population and parents were grown at Pullman, WA, and Bozeman, MT, during the 1997 and 1998 growing seasons. In both locations, each plot consisted of a 3-m row with 30 cm between rows and was planted in two replications in a randomized complete block design. The soil type was a Palouse series (fine-silty, mixed mesic Pachic Ultic Haploxerolls) in Pullman and Amsterdam silt loam (fine silty, mixed Typic haploborolls) in Bozeman. Planting dates were April at Pullman, and May at Bozeman; harvest dates were in August at both locations. Crop management practices were standard for spring wheat production in both locations. At Pullman, the average monthly precipitation from May through August was 3.02 cm (with a range of 0.94 cm in August to 7.19 cm in May). At Bozeman, the average monthly precipitation from May through August was 4.88 cm (with a range of 2.54 cm in May to 10.21 cm in June).

Estimation of PPO Activity

L-DOPA and L-Tyrosine Assays

The procedure for estimating PPO activity followed that of Morris et al. (1998) and Anderson and Morris (2001). A 1.5-mL aliquot of 5 m*M* L-DOPA in 50 m*M* MOPS [3-(*N*-morpholino) propanesulfonic acid] solution, pH 6.5, was added to five seeds in a 2.0-mL microcentrifuge tube. The tubes were rotated for 1.0 h to allow the reaction to take place. Absorbance was measured on 1 mL of the incubated solution at 475 nm with a Shimadzu Biospec-1601 spectrophotometer (Shimadzu Corporation, Columbia, MD). L-Tyrosine assay was carried out according to McCaig et al. (1999) with some modifications. A 1.5-mL aliquot of substrate solution containing 10 m*M* L-tyrosine (disodium salt), 100 m*M* Tris-HCl, pH 9.0, and 2 g L^{-1} Tween 80 was added to a microcentrifuge tube containing 5 seeds. The tubes were rotated for 1.0 h at room temperature to allow the reaction to take place. Absorbance was measured on 1.0 mL of the incubated solution at 405 nm. Seeds from the cultivars Klasic (hexaploid hard

white spring wheat, high PPO) and Renville (spring durum wheat, low PPO) were included with every run as controls to check the consistency of each experiment for both assays.

Statistical Analysis

PPO activity within each population and environment was analyzed by analysis of variance (ANOVA) with the RILs considered a random effect. A combined ANOVA over environments was conducted for each population. The nature of the RIL × environment interaction (whether because of the magnitude of the difference among RILs or changes in the ranking of RILs) was explored by assigning ranks to individuals within environments, then conducting ANOVA for differences among RIL ranks over environments. This procedure is equivalent to the Kruskal-Wallis *k*-sample test (SAS Institute, 1988). A positive *k*-sample test indicates that rank changes are a significant contributor to the RIL × environment interaction. Broad-sense heritability for L-DOPA and L-tyrosine was calculated on a trait mean basis for RILs across environments (Knapp et al., 1985). Transgressive segregation among RILs and parents was tested at $\alpha = 0.05$. The PROC GLM procedure of SAS (SAS Institute, 1988) was used for the above statistical analyses. Linkage analysis between molecular markers and PPO activity was performed by Map Manager QTX (Manly and Olson, 1999; Manly, 2000). Existing RFLP and microsatelite maps for each of the populations (Campbell et al., 1999, 2001; Marino et al., 1996; Nelson et al., 1995a,b,c; Van Deynze et al., 1995) were used. Linkage was determined through an additive regression model, $mY_j = b_0 + b1X_i1 + e_i$, where b_o denoted the intercept, X_i *1* denoted the marker allele of a given RIL at the *i*th locus and mY_j denoted mean PPO activity of the same RIL over locations. A QTL was defined as a marker that had significant association (LOD > 2.4, equivalent to $P < 0.001$) with the trait value in individual and overall environments. An estimate of the percentage of the total phenotypic variation explained by each QTL marker was obtained from the model R^2 values from regression analysis.

RESULTS

L-DOPA and L-Tyrosine Assays

The L-DOPA assay developed in our laboratory (Morris et al., 1998; Anderson and Morris, 2001) has consistently given a dependable measure of PPO activity of wheat seeds. A negative correlation has been found between PPO activity and noodle brightness, i.e., brighter noodle color (greater L* value) corresponds with lower PPO activity. The check cultivars, Klasic (high PPO) and Renville (low PPO), gave average PPO activities (mean ± SD) of 1.00 ± 0.25 AU and 0.05 ± 0.04 AU, respectively ($n = 62$) for the L-DOPA assay. For the L-tyrosine assay, the check cultivars Klasic and Renville gave average PPO activities of 0.99 ± 0.09 and 0.11 ± 0.02 AU, respectively ($n = 25$).

M6/Opata 85

The M6/Opata 85 RIL population had a nearly normal distribution of PPO activity for the 1997 and 1998 growing seasons for both substrates (Fig. 1A). Generally, higher PPO activity was recorded for the L-tyrosine assay. The parents M6 and Opata 85 had similar PPO activities for each substrate (0.21 and 0.19 AU for L-DOPA and 0.37 and 0.36 AU for L-tyrosine, respectively). The population means for PPO activities for the 1997 growing season were 0.23 ± 0.05 AU for L-DOPA, and 0.36 ± 0.07 AU for L-tyrosine, whereas the population means for the 1998 growing season were 0.21 ± 0.05 AU for L-DOPA and 0.38 ± 0.07 AU for L-tyrosine. The range of PPO activities for the whole population was 0.065 to 0.47 AU for L-DOPA and 0.22 to 0.61 AU for L-tyrosine. The ranges of values indicated that both parents contain alleles associated with higher and lower PPO activity. The LSD test at $\alpha = 0.05$ confirmed the presence of transgressive segregation within the progeny (data not shown). ANOVA showed a highly significant difference among RILs, and significant RIL × environment interaction (Table 1). The *k*-test revealed significant changes in RIL rankings between the 1997 and 1998 growouts, indicating a marked environmental effect. Broad-sense heritability for the M6/Opata 85 population was 0.65 (with a 95% confidence interval of 0.52–0.74) for L-DOPA, and 0.64 (with a 95% confidence interval of 0.51–0.74) for L-tyrosine.

In the 1998 growout, two QTL markers were highly significantly associated with L-DOPA PPO activity in the M6/Opata 85 population (Table 2). The marker *Xfba314* located on chromosome 2D explained 23% of the phenotypic variation (Table 2). The marker *Xfbb189*, mapped to chromosome 7D, showed close association with L-DOPA PPO activity at an LOD score of 2.5, and was contributed by the Opata 85 parent. The above markers were not significantly associated with L-DOPA PPO activity for the 1997 growout (data not shown). However, when linkage analysis was conducted on the RIL means over both locations, a significant association was discovered between the QTL marker *Xfba314* and L-DOPA PPO activity (Fig. 2, Table 2). For the L-tyrosine assay, markers significantly associated with PPO activity were not found.

NY18/CC

The NY18 parent had a lower mean PPO activity than the Clark's Cream parent for both substrates, consistent with the L-tyrosine results of Udall (1997). The PPO activities for the population ranged from 0.08 to 1.00 AU for L-DOPA, and from 0.35 to 1.13 AU for L-tyrosine. The 1994 greenhouse growout had a normal distribution, whereas the 1994 field growout had slightly more lines with low PPO activities and the distribution was not normal (Fig. 1B). The PPO activities for the 1998 Central Ferry growout were normally distributed, but were much higher than PPO activities for the two sets of the population grown in 1994 in Wooster (Fig. 1B), especially for the L-DOPA assay. The mean L-DOPA PPO activities were 0.42 ± 0.13 AU for the 1994 greenhouse, 0.41 ± 0.16 AU for the 1994 field growout, and 0.68 ± 0.13 AU for the 1998 field growout. The mean L-tyrosine PPO activities were 0.57 ± 0.14 AU for the 1994 greenhouse, 0.40 ± 0.20 AU for the 1994 field growout, and 0.69 ± 0.12 AU for 1998 field growout. ANOVA revealed significant differences among RILs and environments for both substrates (Table 1). The broad-sense heritability for the three environments was

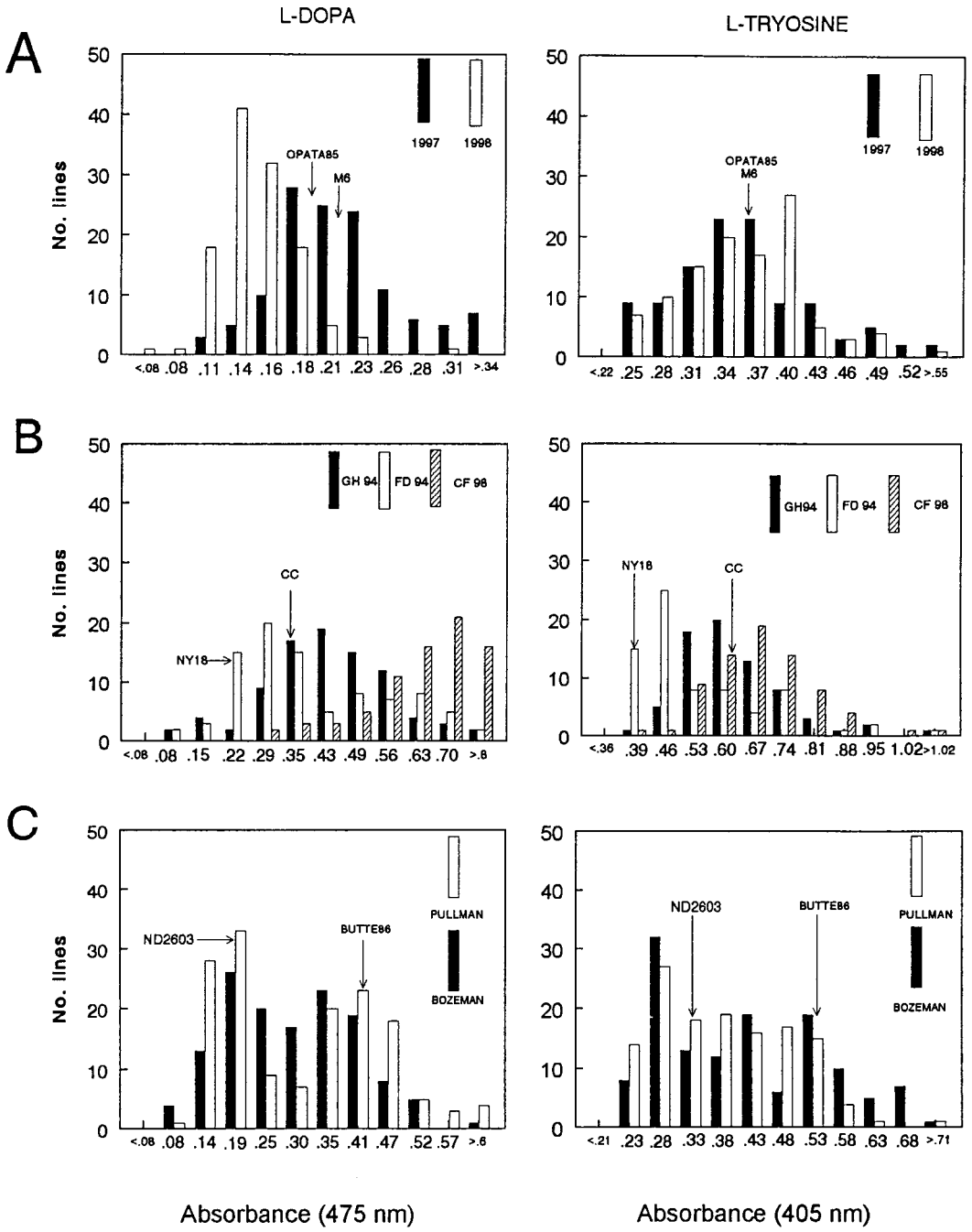

Fig. 1. Distribution of PPO activity (L-DOPA and L-tyrosine) for M6/Opata 85 (A), NY18/CC (B), and ND2603/Butte 86 (C) recombinant inbred lines. Arrows indicate parental means. CF98 is Central Ferry, 1998; FD94 is Wooster, field, 1994; and GH94 is Wooster, greenhouse, 1994. For the NY18/CC population, the parental values for 1998 were not available. The averages indicated are based on the 1994 field and greenhouse growouts. In 'C' the PPO activity is the average for the 1997 and 1998 data for both Pullman and Bozeman.

0.58 (with a 95% confidence interval of 0.41–0.70) for L-DOPA, and 0.46 (with a 95% confidence interval of 0.24–0.61) for L-tyrosine.

Markers significantly ($P < 0.001$) associated with PPO activity in the NY18/CC population were not detected for 1994 and 1998 field growouts for either substrate. Two QTL markers (*Xrz900b* and *Xbcd543*) were significantly associated with L-DOPA PPO activity at LOD scores of 2.5 for the Wooster 1994 greenhouse growout (Table 2). Similarly, three QTL markers (*Xbcd307c*, *Xwmg546a*, and *Xuaz145c*) were significantly associated with PPO activity for L-tyrosine for the 1994 greenhouse growouts (Table 2). Four of these five markers are located on chromosome 2AL, the fourth is located on chromosome 2B. For the combined analysis over locations, the markers *XksuG12a*, *Xrz753DrI*, and *Xbcd809* showed an association with L-DOPA PPO activity at LOD scores of 2.7, 2.6, and 2.4, respectively (Table 2). For L-tyrosine, the combined analysis over three locations revealed a significant association of the QTL

Table 1. Analysis of variance for polyphenol oxidase activities among three recombinant inbred line populations of wheat.

Source	df	Mean square	
		L-DOPA	L-tyrosine
M6/Opata 85			
Year	1	0.0303****	0.0292**
Rep (year)	2	0.0056**	0.0386****
Genotype	108	0.0088****	0.017****
Genotype × year	108	0.0031****	0.0061**
Residual	219	0.0011	0.004
NY18 × CC			
Location	2	1.6749****	1.6043***
Genotype	77	0.0282****	0.0361****
Residual	148	0.0117	0.0197
ND2603/Butte 86			
Location	1	0.0045 NS	3.1716****
Rep (location)	2	0.0133**	0.0441**
Genotype	133	0.0544****	0.0613****
Genotype × location	133	0.0088****	0.012****
Residual	262	0.0087	0.012

** Indicates significance at $P < 0.01$.
*** Indicates significance at $P < 0.001$.
**** Indicates significance at $P < 0.001$.
NS = not significant.

markers *Xbcd307c* and *Xuaz145c* with PPO activity (Table 2).

ND2603/Butte 86

This population had a bimodal distribution with a wide range of values for the L-DOPA assay (Fig. 1C). For the L-tyrosine assay, the distribution was not bimodal. The average PPO activity for the parents was 0.19 AU for L-DOPA and 0.32 AU for L-tyrosine for ND2603; and 0.41 AU for L-DOPA and 0.55 AU for L-tyrosine for Butte 86. The minimum and maximum PPO activities for the RIL population were 0.08 and 0.68 AU for the L-DOPA assay, and 0.22 and 0.89 AU for the L-tyrosine assay. The overall average PPO activity was 0.33 AU for L-DOPA assay and 0.46 AU for L-tyrosine assay. The RIL effect was highly significant based on ANOVA. However, there was also significant RIL × environment interaction (Table 1). The *k*-test did not show significance for location L-DOPA PPO activity, which means that ranks of RILs did not change appreciably over locations, and the interactions were due primarily to changes in the degree of difference among RILs. However, the *k*-test was significant for L-tyrosine indicating a change of ranks for RILs across environments. Significant transgressive segregation was observed for the population (LSD $\alpha = 0.05$). The broad-sense heritability was 0.84 (with a 95% confidence interval of 0.79–0.88) for L-DOPA, and 0.80 (with a 95% confidence interval of 0.74–0.85) for L-tyrosine.

Very few QTL markers are currently mapped on the ND2603/Butte 86 RIL population. The marker Xbcd907.RV.1 located on chromosome 3BS showed an association with PPO activity at an LOD score of 2.1 for L-DOPA. QTL markers associated with PPO activity were not detected for L-tyrosine activity.

DISCUSSION

The objective of this study was to investigate the distribution, chromosome location and number of loci for wheat PPO by means of three populations of recombinant inbred lines and two substrates. A nearly normal distribution was observed for PPO activities for the M6/Opata 85 population. The NY18/CC populations had a nearly normal distribution for the 1994 greenhouse and 1998 field growouts. However, the 1998-grown RIL data set had much higher PPO activity than that of the 1994 RIL data sets for both substrates. The higher PPO activity for the 1998 Central Ferry growout for the NY18/CC RIL population for both substrates clearly indicates a large environmental effect for the expression of PPO activity in this population. When examined visually, the Central Ferry grown grain was larger and plumper than that from the Wooster greenhouse and field environments. Differences in PPO activities among the parents in the M6/Opata 85 and the NY18/CC populations were slight, but the progeny exhibited a wide range of values, and transgressive segregation was evident. Therefore, PPO activity is controlled by multiple loci for the two RIL populations. Transgressive segregation (when progeny traits exceed those of the parents) is also an indicator that phenotypes were derived through an additive combination of parental alleles. The bimodal distribution and the larger difference between parental values for the ND2603/Butte 86 population indicated that a single

Table 2. DNA markers significantly associated† with polyphenol oxidase activity (L-DOPA and L-tyrosine) in three recombinant inbred line populations of wheat.

Marker	LOD‡	R^2	Chromosome	High parent
M6/Opata 85 (L-DOPA, Tule Lake, 1998)				
Xfba314	3.2	23	2D	M6
Xfbb189	2.5	18	7D	Opata 85
M6/Opata 85 (L-DOPA, mean over Tule Lake 1997 and 1998)				
Xfba314	3.2	23	2D	M6
NY18/CC (L-DOPA, Wooster Greenhouse 1994)				
Xrz900b	2.5	14	2AL	NY18
Xbcd543	2.5	13	2B	NY18
Xcdo64	2.4	13	2AS, L	CC
Xcdo1376	2.3	12	2B	NY18
NY18/CC (L-DOPA, Wooster Field 1994)				
Xbcd907a	2.3	13	3S	CC
XksuG12a	2.2	12	6BS, L	CC
NY18/CC (L-DOPA, mean over Wooster Greenhouse and Field 1994)				
Xbcd907a	2.4	14	3S	CC
XksuG12a	2.4	13	6BS, L	CC
NY18/CC (L-DOPA mean over Wooster Greenhouse and Field 1994, Central Ferry, 1998)				
XksuG12a	2.7	14	6BS, L	CC
Xrz753DrI	2.6	13	2BS, L	CC
Xbcd809	2.4	13	3DL	NY18
NY18/CC (L-tyrosine, Wooster Greenhouse 1994)				
Xbcd307c	3.1	16	2AL-1	NY18
Xmwg546a	2.5	13	2AL-1	NY18
Xuaz145c	2.5	13	2AL-1	NY18
Xbcd1428a	2.3	12	2ASL-1	CC
NY18/CC (L-tyrosine, mean over Greenhouse and Field 1994, Central Ferry, 1998)				
Xuaz145c	2.5	13	2AL-1	NY18
Xbcd307c	2.4	12	2AL-1	NY18
Xcdo373	2.3	12	2AL-1	NY18
Xbcd120a	2.2	11	2ASL-1	CC
ND2603/Butte 86 (mean over Pullman and Bozeman, 1998)				
Xbcd907.RV.1	2.1	11	3BS	ND2603

† The 1997 data set for M6/Opata 85 identified no loci significantly linked to PPO activity. QTLs were not identified for the L-tyrosine assay for the M6/Opata 85 population. No significant QTL was identified for the 1994 and 1998 field data sets for the NY18/CC population for either PPO assay. 2AL-1 implies chromosome 2AL, linkage group 1.
‡ LOD is the logarithm of the odds ratio of the probabilities that the data are linked versus not linked. LOD of 2.4 is equivalent to $P < 0.001$.

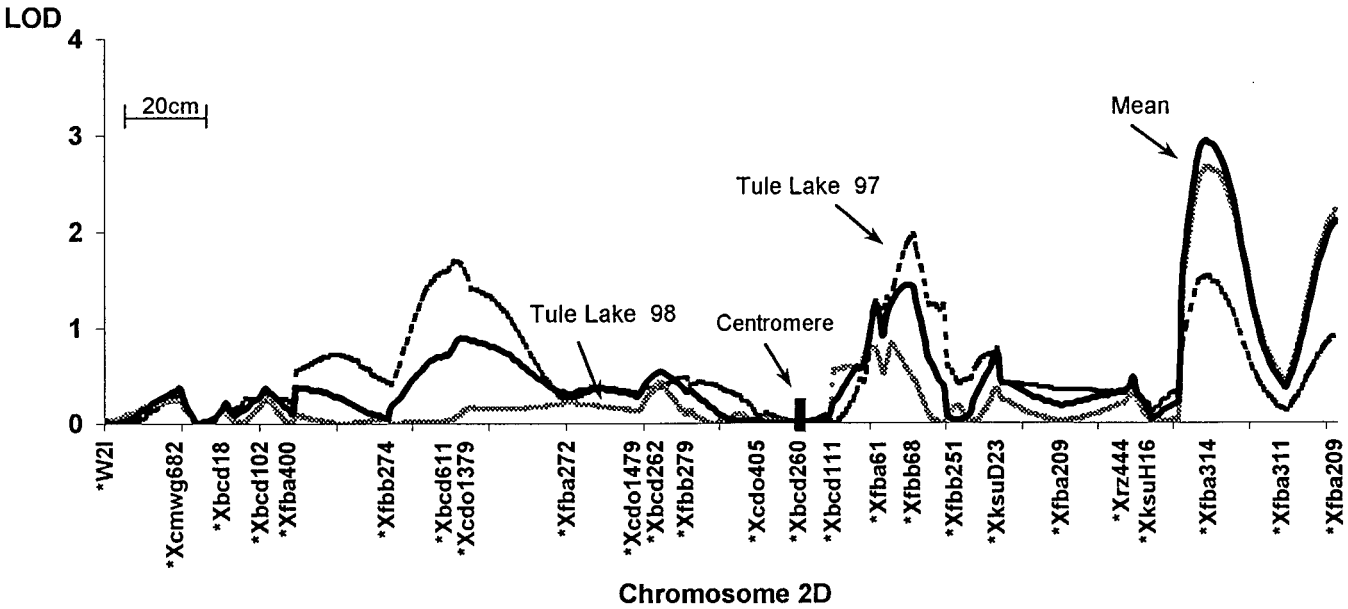

Fig. 2. LOD scores of association between PPO activity and molecular markers on wheat chromosome 2D.

major gene influenced PPO activity in that population for the L-DOPA assay. This gene may or may not be present in the other two populations.

For the M6/Opata 85 population, one marker significantly associated with PPO activity was detected on chromosome 2D by the L-DOPA assay. For the NY18/CC RIL population, markers significantly associated with PPO activity were found for the 1994 Wooster greenhouse environment for both substrates (Table 2). However, QTL markers were not detected for the 1994 and 1998 field growouts for either substrate. For the combined analysis of the three environments, three QTL markers for L-DOPA, and two different QTL markers for L-tyrosine (Table 2) showed significant associations with PPO activity. Both parents contributed to the expression of the trait. The ND2603/Butte 86 population has not been widely used for mapping, so few markers are available. An LOD score of >2.0 has been used as a threshold for QTL associations in some studies (Grandillo and Tanksley, 1996; Xiao et al., 1996). Therefore, the QTL marker *Xbcd907.RV.1*, that revealed an association within the ND2603/Butte 86 population at a LOD score of 2.1, may be a useful marker. For a given substrate and population, the QTL markers significantly associated with PPO activity in one environment also showed an association with PPO activity in other environments (albeit not statistically significant), showing the consistency of the markers. The measurement of PPO activity with substrates such as L-DOPA and L-tyrosine, although useful and simple, is clearly influenced by environment. DNA molecular markers are stable and independent of the environment. Additionally, QTL markers are useful in describing the loci controlling quantitative traits. Low PPO wheat genotypes could be selected using closely linked QTL markers instead of multiple PPO measurements over years and locations. Furthermore, map-based cloning system can be used to identify PPO genes, once closely linked QTL markers are identified.

There was a significant location × RIL or year × RIL interaction in each population, suggesting a differential effect of environment on expression of PPO activity with both substrates. A pronounced effect of environmental variation on wheat PPO activity has also been reported in other studies (McCaig et al., 1999; Park et al., 1997). Significant positive correlations were observed between L-DOPA (diphenol) and L-tyrosine (monophenol) for all but the Central Ferry-grown NY18/CC population (Table 3). For the 1998 growout of NY18/CC, the L-DOPA values were markedly higher, whereas the L-tyrosine values were only moderately higher, thus contributing to a lower correlation between assays (Table 3). Variations in substrate specificity are well known among the class of enzymes referred to as PPO (Lamkin et al., 1981; Lee and Whitaker, 1995; Taneja and Sachar, 1974). According to Taneja et al. (1974), wheat PPO activity was affected by substrate, genotype, and maturity of the grain. Six to 10 protein bands were observed when catechol (*o*-diphenol) was used as substrate, whereas, only one protein band was noticed when using L-tyrosine (monophenol). For the NY18/CC RIL population, different QTL markers were identified for both assays, indicating substrate specificity. Udall (1997) used the

Table 3. Spearman and Pearson correlation coefficients relating L-tyrosine to L-DOPA assay results by recombinant inbred line (RIL) population and environment or location.

RIL	Spearman	Pearson
M6/Opata 85		
1997	0.57****	0.54****
1998	0.44****	0.38****
NY18/CC		
Greenhouse 1994	0.58****	0.61****
Field 1994	0.73****	0.80****
Central Ferry 1998	0.24NS	0.29NS
ND2603/Butte 86		
Bozeman	0.78****	0.76****
Pullman	0.84****	0.82****

**** Significant at $P = 0.0001$.
NS = not significant.

NY18/CC RIL population for QTL mapping and found the marker *Xcdo373* to be significantly associated with L-tyrosine activity. In our study, *Xcdo373* was not significantly associated with PPO activity as determined by L-DOPA, but showed a strong association with PPO activity when L-tyrosine was used (Table 2). Both substrates can be used to measure PPO activity, but L-DOPA dissolves readily, is stable at neutral pH, and is not toxic to seeds (Anderson and Morris, 2001). Therefore, the difference in these results could be accounted for by the procedure used to measure PPO activity as well as environmental influence.

The chromosome location of a wheat PPO gene has been suggested to be on chromosome 2 (Jimenez and Dubcovsky, 1999; Udall, 1997; Anderson and Morris, 2001). In this study, most of the QTL markers that explained greater amounts of variation are also on chromosome 2, in agreement with other reports. Although a major QTL could be assigned to chromosome 2D for PPO, minor QTLs could be located on other chromosomes as our study suggests.

CONCLUSIONS

The wheat PPO enzyme is implicated in causing browning of noodles, chappatis, and other wheat products. Analysis of three wheat RIL populations revealed polygenic inheritance in two populations and monogenic inheritance in a third population. There was relatively low to medium correlation between the L-DOPA and L-tyrosine assays in PPO activity which indicates substrate specificity. Both RIL and environment affected PPO activity. We have identified a QTL marker significantly associated with wheat PPO activity on chromosome 2D in the M6/Opata 85 mapping population. QTL markers significantly associated with PPO activity were also detected on chromosomes 2A, 2B, 3B, 3D, and 6B at LOD scores of >2.4. Identification of QTL markers associated with PPO activity has the potential to accelerate selection for improved end-use quality and to resolve in greater detail the genetic basis of this important trait.

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Association of the Brazil Nut Protein Gene and Kunitz Trypsin Inhibitor Alleles with Soybean Protease Inhibitor Activity and Agronomic Traits

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ABSTRACT

The use of soybean [*Glycine max* (L.) Merr.] in animal feed without heat treatment may be possible by reducing protease inhibitors. The objectives of this study were to determine to what extent soybean protease inhibitors would be reduced genetically by combining the Brazil nut protein (BNP) gene from the transgenic line BX4P9341B6 with the *ti* allele for the Kunitz trypsin inhibitor from the cultivar Kunitz and their effect on agronomic traits. Soybean seed from 42 F_2-derived lines from the cross BX4P9341B6 × Kunitz were evaluated in replicated trials in 1995 for trypsin inhibitor (TI), chymotrypsin inhibitor (CI) activity, and agronomic traits. There were six lines homogeneous for presence of the BNP gene and the *Ti* allele (BNP+, *Ti*), 18 lines homogeneous for presence of the BNP gene and the *ti* allele (BNP+, *ti*), six lines homogeneous for absence of the BNP gene and presence of the *Ti* allele (BNP−, *Ti*), and 12 lines homogeneous for absence of the BNP gene and presence of the *ti* allele (BNP−, *ti*). The mean TI activity of the BNP+, *ti* lines was 85.1% less than for the BNP−, *Ti* lines representative of conventional soybean cultivars. The mean CI activity of the BNP+, *ti* lines was 61.4% less than the BNP−, *Ti* lines. The means of the four genotypic classes were not significantly different ($P > 0.05$) for seed yield, maturity, lodging, and protein content. It should be possible to develop high-yielding cultivars with the BNP+, *ti* genotype that have major reductions in TI and CI activity.

Soybean is a major source of protein meal in the world. Protease inhibitors, including the Kunitz trypsin inhibitor (KTI) and chymotrypsin inhibitor (CI), are responsible for the inferior nutritional quality of unheated or incompletely heated soybean meal. Ingestion of raw soybeans causes pancreatic hypertrophy (Booth et al., 1960). Proper heat processing is required to destroy protease inhibitors. Excessive heat treatment may lower amino acid availability and reduce animal weight gain (Herkelman and Cromwell, 1990; Lee and Garlich, 1992). Soybean lines with reduced protease inhibitor content could reduce or eliminate the need for expensive heat treatment and lessen the chance of lowering amino acid availability.

Accessions in the USDA soybean germplasm collection have been screened by polyacrylamide gel electrophoresis for the presence or absence of electrophoretic forms of soybean trypsin inhibitors (SBTI). Five electrophoretic forms have been discovered (Hymowitz and Hadley, 1972; Orf and Hymowitz, 1977; Orf and Hymowitz, 1979; Singh et al., 1969; Quigyan et al., 1995). The genetic control of four forms, Ti^a, Ti^b, Ti^c, and Ti^d, has been reported as a codominant multiple allelic series at a single locus (Singh et al., 1969; Hymowitz and Hadley, 1972; Orf and Hymowitz, 1977; Quigyan et al., 1995). Orf and Hymowitz (1979) found that the fifth form does not exhibit a SBTI-A_2 band and is inherited as a recessive allele designated *ti*. They also found that crude seed protein from seeds that lacked the SBTI-A_2 band had a 30 to 50% reduction in trypsin inhibitor (TI) activity compared with 'Amsoy 71' that has the SBTI-A_2 band.

Scientists at Pioneer Hi-Bred International, Inc. observed that the introduction into soybean of a gene from the Brazil nut tree [*Bertholletia excelsa* (Castanheira) Humb. & Bonpl.] encoding higher levels of methionine-rich 2S seed storage protein resulted in a reduction in TI and CI (Beach et al., 1995). One objective of this study was to determine if TI and CI activity could be

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